ORIGINAL ARTICLE

Mast Cell Activation Profile and $T_{FH}13$ Detection Discriminate Between Food Anaphylaxis and Sensitization

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Abstract

Background: The prevalence of food allergy (FA) has increased significantly, and the risk of developing anaphylaxis is unpredictable. Thus, discriminating between sensitized patients and those at risk of a severe reaction is of utmost interest.
Objective: To explore the mast cell (MC) activation pattern and the presence of T follicular helper (T_{FH}) 13 cells in sensitized patients and patients who experience food anaphylaxis.
Methods: Patients sensitized to lipid transfer protein (LTP) were classified as being at risk of anaphylaxis or sensitized depending on the symptoms elicited by the LTP-containing food. $CD34^+$-derived MCs were obtained from patients and controls, sensitized with pooled sera, and challenged with Pru p 3 (peach LTP). Degranulation, prostaglandin (PG) D2, and cytokine/chemokine release were measured. The $T_{FH}13$ population was examined using flow cytometry of the peripheral blood of all groups. In parallel, LAD2 cells were activated in the same way as patients' MCs.
Results: A distinguishable pattern of MC activation was found in anaphylaxis patients but not in sensitized patients. Robust degranulation, PGD2, and release of IL-8 and granulocyte-macrophage colony-stimulating factor were more frequent in anaphylaxis patients, whereas secretion of TFG-ß and CCL2 was more frequent in sensitized patients. Anaphylaxis patients also had a larger $T_{FH}13$ population. The MC activation profile was dependent on the sera rather than the MC source. Consistent with this observation, LAD2 cells reproduce the same pattern as MCs from anaphylactic and sensitized patients.
Conclusion: The distinct profile of MC activation makes it possible to discriminate between patients at risk of an anaphylactic reaction and sensitized patients. Pooled sera may determine MC activation independently of MC origin. Besides, the presence of $T_{FH}13$ cells in anaphylaxis patients points to an essential role for the affinity of IgE.

Key words: Anaphylaxis. Food allergy. IgE. Inflammation. Mast cells. $T_{FH}13$.

Resumen

Antecedentes: La prevalencia de alergia alimentaria (AA) se ha incrementado de forma significativa, y el riesgo de desarrollar una anafilaxia es impredecible. Por este motivo, el poder discriminar entre individuos sensibilizados y aquellos en riesgo de desarrollar una reacción grave es de gran interés.
Objetivo: Comparar el patrón de activación mastocitaria y la presencia de linfocitos T foliculares helper (T_{FH}) 13 en individuos sensibilizados y con alergia alimentaria.
Metodología: Pacientes sensibilizados a la proteína de transferencia de lípidos (LTP) se clasificaron en dos grupos, anafilaxia y sensibilizados, en función de los síntomas inducidos por el consumo de alimentos con LTP. Se obtuvieron mastocitos derivados a partir de progenitores $CD34^+$, tanto de pacientes como de controles, se sensibilizaron con el suero de los diferentes individuos, y se estimularon con Pru p 3 (LTP de melocotón). Se evaluó la desgranulación, los niveles de PGD2 y la liberación de citocinas/quimioquinas. La población $T_{FH}13$ se examinó en sangre periférica mediante citometría de flujo en todos los grupos. En paralelo, células LAD2 se activaron del mismo modo que los mastocitos de los pacientes.
Resultados: Se observó un patrón de activación mastocitaria diferencial entre los pacientes con anafilaxia y los sensibilizados. La desgranulación, la producción de PGD2, IL-8 y GM-CSF fue superior en el grupo de anafilaxia, mientras que la producción de TFG-ß y

CCL2 era superior en pacientes sensibilizados. De forma concomitante, los pacientes con anafilaxia presentaron una población mayor de $T_{FH}13$. El perfil de activación mastocitaria fue dependiente del suero del paciente, más que del origen del mastocito. Igualmente, este patrón se puedo observar en el modelo de LAD2, tanto el observado en mastocitos de pacientes con anafilaxia como en los sensibilizados. *Conclusion:* Los pacientes con anafilaxia se pueden diferenciar de los sensibilizados gracias a la presencia de un patrón de activación mastocitaria distinto. Además, la mayor presencia de linfocitos $T_{FH}13$ en los pacientes con anafilaxia sugiere un papel destacable de la afinidad de la IgE en el desarrollo de la anafilaxia.

Palabras clave: Anafilaxia. Alergia alimentaria. IgE. Inflamación. Mastocitos. $T_{FH}13$.

Summary box

- **What do we know about this topic?**
  The prevalence of food allergy has increased significantly in recent decades. There are currently no biomarkers to identify which food-sensitized patients will develop anaphylaxis.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  The MC activation profile and detection of $T_{FH}13$ make it possible to identify patients at risk of anaphylaxis from sensitized patients, facilitating risk stratification in a complex model of food allergy such as LTP.

Introduction

The frequency of food anaphylaxis has increased dramatically in recent decades [1-3]. However, the main prophylactic strategy is still limited to food avoidance. Diagnosis of food allergy (FA) is frequently challenging owing to the difficulties differentiating between sensitization and true allergy [4-6]. This is particularly problematic in patients sensitized to panallergens related to anaphylaxis, such as lipid transfer proteins (LTPs) [7,8]. Thus, oral challenge tests continue to be the gold standard, although tools for accurate risk assessment are limited. Consequently, in some cases, patients may follow unnecessarily restricted diets that significantly impact their quality of life [9].

In recent years, the basophil activation test (BAT) was shown to help distinguish clinically relevant from irrelevant sensitization in FA [10-12]. Since mast cells (MCs) are considered the primary effector cells of allergy [13], an MC activation test (MAT) was developed in parallel and has already improved the diagnosis of IgE-mediated peanut allergy [14,15]. MCs are commonly obtained from peripheral blood by isolation and differentiation of progenitors in vitro [15-19]. Other approaches involve the use of MCs derived from induced pluripotent stem cells [20], and, in a less laborious and time-consuming procedure, the human MC line LAD2 has also yielded good results [21].

Our understanding of the immune basis of FA has increased in recent years. Studies of food-allergic patients and murine models point to the affinity of IgE as a key factor related to MC degranulation and anaphylaxis [22-25]. T follicular helper (T_{FH}) cells direct the affinity and isotype of antibodies synthesized by B cells. Moreover, the nature of signals that switch from low to high affinity may differ [26,27]. T_{FH} cell–derived interleukin 4 (IL-4) is necessary for production of IgE; however, IL-13–producing T_{FH} cells are induced by allergens [28,29]. These $T_{FH}13$ cells have a different cytokine profile (IL-13^{hi}IL-4^{hi}IL-5^{hi}IL-21^{lo}) and are required to produce high- but not low-affinity IgE. Since $T_{FH}13$ are necessary for anaphylactic reactions, they may facilitate identification of patients at risk of severe reactions [30].

Accordingly, release of MC mediators may depend on the affinity of IgE. High-affinity IgE induces stronger degranulation and more marked cytokine release than the low-affinity IgE, resulting in more elevated chemokine secretion in the latter [23,31]. Thus, secretion of cytokines and chemokines may differ in FA [32].

This study aims to identify factors that can differentiate patients at risk of anaphylaxis from those who are only sensitized. In 2 phenotypes of LTP-sensitized individuals, ie, patients with food anaphylaxis and sensitized individuals (asymptomatic), we compared (*1*) MC activation induced by peach LTP (Pru p 3) and (*2*) the presence of $T_{FH}13$ cells in peripheral blood. Our results show that serum samples from anaphylaxis patients induce distinguishable MC activation patterns. The $T_{FH}13$ cell population is more abundant in anaphylaxis patients than in sensitized individuals, suggesting its potential use as a biomarker of the risk of severe reactions.

Material and Methods

Study Population

Patients were recruited at the Allergy Department of Hospital Clínic, Barcelona, Spain. Informed consent was obtained from all participants. The study was approved by the local ethics committee.

The patients recruited were sensitized to peach LTP-Pru p 3 with specific IgE (sIgE) levels [3] >0.10 kU_A/L (ImmunoCAP, Thermo Fisher Scientific) and no other sensitization identified. They were classified into 2 groups depending on the severity of the reaction upon peach ingestion, as follows: (*1*) anaphylaxis patients with a convincing history of anaphylaxis and (*2*) sensitized patients with no symptoms. The oral challenge was not performed in anaphylaxis patients [33], and a recent history of tolerance to peach was required in the sensitized group. Healthy volunteers with no respiratory or food allergies were also recruited as controls.

To carry out this study, the patients and healthy volunteers recruited were divided into 2 cohorts for the in vitro studies: (*1*) a cohort to generate pooled sera for MC activation, PGD2 secretion, and cytokine analysis assays; and (*2*) a cohort to generate MCs and to detect $T_{FH}13$.

Total IgE and Pru p 3–specific IgG4 (sIgG4) were measured using the ImmunoCAP System (Thermo Fisher Scientific).

$CD34^+$-Derived Mast Cell Generation

A total of 15 individuals were recruited: 6 with anaphylaxis, 5 sensitized, and 4 healthy volunteers (demographic and clinical characteristics are presented in Table 1). MCs from these groups were obtained from 100 mL of peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation with Histopaque (Sigma) following a protocol described elsewhere [15,16]. Briefly, blood was diluted with PBS (Lonza Bioscience), layered over Histopaque, and centrifuged at room temperature at 400g for 20 minutes. PBMCs were collected, washed, and incubated with MACS Buffer (0.5% BSA; 2 mM EDTA; 50 mL PBS) and the CD117 Microbead Kit, human (Miltenyi Biotec). $CD117^+$ (or KIT) cells were selected using a magnetic field with LS columns (Miltenyi Biotec) and suspended with MC culture medium (StemPro-34 Medium, Thermo Fisher) supplemented with 1% penicillin-streptomycin (Lonza Bioscience), 1% L-glutamine (Lonza Bioscience), recombinant human (rh)

Table 1. Patients Selected for Detection of CD34+-Derived Mast Cells and $T_{FH}13$.

Patient ID	Group	Sex	Age	Pru p 3 IgE, kU_A/L	Tryptase, µg/L	Symptoms
1	Anaphylaxis	Male	30	24.7	2.2	OAS with sunflower seed, pistachio, almond, peanut, walnut, hazelnut, lettuce, lentil, apple, grapefruit, avocado, banana, orange. Anaphylaxis (urticaria, hypotension, bronchospasm) without cofactor with nuts, sunflower seeds, peach juice, vegetable mix.
2	Anaphylaxis	Male	50	1.05	2.3	Urticaria and angioedema with seeds. Anaphylaxis (urticaria, angioedema, and bronchospasm) with apple. Contact urticaria with peach. OAS with several nuts (hazelnut, walnut, peanut).
3	Anaphylaxis	Male	55	7.96	4.5	Anaphylaxis (urticaria, abdominal pain, vomiting, bronchospasm) without cofactor with peach, hazelnut, and walnut.
4	Anaphylaxis	Female	42	8.76	ND	Urticaria with ingestion of mixed vegetables, apple. Gastrointestinal symptoms with green beans. Anaphylaxis (urticaria, angioedema, bronchospasm) with almonds and cofactor (NSAID).
5	Anaphylaxis	Female	32	13.00	2.4	Anaphylaxis (urticaria, bronchospasm, hypotension) without cofactor with peach and walnut.
6	Anaphylaxis	Male	56	16.50	5.4	OAS with ingestion of peanut, corn, walnut. Gastrointestinal symptoms with mixed vegetables and peach. Urticaria and angioedema with peach and nuts. Anaphylactic shock (urticaria, lingual angioedema, hypotension, loss of consciousness) with nectarine and cofactor (physical exercise).
7	Sensitized	Male	63	0.43	5.7	Asymptomatic sensitization
8	Sensitized	Female	46	41.20	3.9	Asymptomatic sensitization
9	Sensitized	Female	37	28.70	6.3	Asymptomatic sensitization
10	Sensitized	Male	48	0.66	5.2	Asymptomatic sensitization
11	Sensitized	Female	74	1.25	4.4	Asymptomatic sensitization
12	Healthy	Male	30	0.03	ND	Healthy individuals
13	Healthy	Female	22	0.03	ND	Healthy individuals
14	Healthy	Male	28	0.02	ND	Healthy individuals
15	Healthy	Male	35	0.03	ND	Healthy individuals

Abbreviations: ND, not determined; NSAID, nonsteroidal anti-inflammatory drugs; OAS, oral allergy syndrome.

IL-6 (50 ng/mL, Immunotools), and SCF (100 ng/mL, Immunotools). rh IL-3 (10 ng/mL, Immunotools) was added at day 0 of culture. MC culture medium was added to the cell culture every 2 weeks. MCs were characterized at week 7.

Characterization of CD34⁺-Derived Mast Cells

To verify morphology, a volume of 5×10^4 cells was centrifuged with Cytospin at 500 rpm for 5 minutes and then stained with May-Grünwald Giemsa.

For MC differentiation analysis, a volume of 5×10^4 cells was taken from culture, blocked, and stained with APC-conjugated anti-FcεRI (BioLegend) and PE-conjugated anti-CD117 (Santa Cruz Biotechnology). Cells were acquired on a FACSCalibur flow cytometer (FACScan, BD Biosciences) and analyzed using FlowJo software, version 10.8.

The β-hexosaminidase assay was performed to check the functionality of CD34⁺-derived MCs. A total of 6×10^4 cells was taken from culture and sensitized overnight with

Table 2. Patients Selected for Preparation of Pooled Sera.[a]

Pool and patient ID	Total IgE, kU_A/L	Pru p 3 IgE, kU_A/L	Ratio sIgE:tIgE	Pru p 3 IgG4, kU_A/L	Ratio sIgG4/sIgE	Symptoms
Anaphylaxis						
1	48.2	11.3	4.26	ND	ND	Anaphylaxis (urticaria, bronchospasm) with pomegranate.
2	117	10.7	10.93	ND	ND	Anaphylaxis (urticaria, bronchospasm, hypotension, loss of consciousness) with tomato and cofactor (NSAID).
3	136	61	2.22	ND	ND	Anaphylaxis (urticaria, vomiting, bronchospasm) with apple and cofactor (physical exercise). OAS with peanut. Contact urticaria with peach.
4	177	9.46	18.71	ND	ND	Anaphylaxis (urticaria, hypotension, bronchospasm) with peach, hazelnut, and peanut. Contact urticaria with peach.
5	139	7.96	17.46	ND	ND	Anaphylaxis (urticaria, diarrhea, bronchospasm) with peanut. Urticaria with corn, peanut, and hazelnut. OAS with peach, tomato, and lettuce.
6	96.6	5.8	16.65	ND	ND	Anaphylaxis (urticaria, bronchospasm) with walnut and cofactor (alcohol). Contact urticaria with peach. OAS with walnut and hazelnut.
7	132	16.5	8.00	ND	ND	OAS with apple, hazelnut. Urticaria and angioedema with tomato. Anaphylaxis (angioedema, diarrhea, bronchospasm, hypotension) with walnut and cofactor (physical exercise).
8	159	17.7	8.98	ND	ND	Gastrointestinal symptoms with lettuce, tomato, green beans. OAS with hazelnut, peanut, and walnut. Anaphylaxis (urticaria, angioedema, hypotension) with walnut and cofactor (NSAID).
Pool	ND	19.80	ND	2.31	8.59	
Sensitized						
9	229	0.88	260.22	ND	ND	Asymptomatic sensitization
10	160	1.25	128.00	ND	ND	Asymptomatic sensitization
11	228	1.64	139.02	ND	ND	Asymptomatic sensitization
12	316	1.22	259.01	ND	ND	Asymptomatic sensitization
Pool	ND	0.98	ND	0.46	2.13	
Healthy						
13	ND	<0.10	ND	ND	ND	Healthy individuals
14	ND	<0.10	ND	ND	ND	Healthy individuals
15	ND	<0.10	ND	ND	ND	Healthy individuals
16	ND	<0.10	ND	ND	ND	Healthy individuals
17	ND	<0.10	ND	ND	ND	Healthy individuals
Pool	ND	<0.10	ND	0.09	0.56	

Abbreviations: ND, not determined; NSAID, nonsteroidal anti-inflammatory drug; OAS, oral allergy syndrome.
[a]All healthy volunteers had Pru p 3 sIgE <0.10 kU_A/L.

0.1 µg/mL biotinylated human IgE (Abbiotec) in triplicate into 96-well plates. Cells were stimulated with 0.4 µg/mL streptavidin (Sigma) for 30 minutes at 37°C. Plates were subsequently centrifuged, and β-hexosaminidase was assayed in the supernatants and cell pellets as described elsewhere [34,35]. Degranulation was expressed as the percentage of β-hexosaminidase recovered from the supernatants compared with total cellular content.

Mast Cell Activation and PGD2 Secretion

We obtained serum samples from 8 anaphylaxis and 4 sensitized patients. As described above, these sera were pooled based on similar ratios of Pru p 3 sIgE to total IgE levels (sIgE:tIgE). The characteristics of the individual donors and pooled sera are shown in Table 2.

Next, a total of 5×10^4 MCs or LAD2 cells, the latter kindly provided by Dr. D. Metcalfe (NIH, Bethesda, MD, USA) [36], was incubated with 10 ng/mL rh IL-4 (Immunotools) for 5 days [37,38] and sensitized overnight with pooled sera from the different groups (Table 2) diluted to obtain a total Pru p 3 sIgE concentration of 1 kU_A/L. Cells were washed and stimulated with 1 µg/mL Pru p 3 (Roxall) for 30 minutes at 37°C. The supernatants were kept at –80°C for later prostaglandin D2 (PGD2) analysis using an ELISA kit (Cayman Chemical) as described elsewhere [39]. Cells were blocked and stained with PE-conjugated anti-CD63 (BD Biosciences). Cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software, version 10.8. Where possible, experiments were performed in duplicate in patients (limitation: low number of cells obtained).

Detection of $T_{FH}13$ Cells

$T_{FH}13$ cells were detected following a previously described protocol [29]. Briefly, PBMCs from patients and healthy volunteers were thawed, and $CD4^+$ T cells were isolated using the EasySep Human $CD4^+$T Cell Enrichment Kit (Stemcell Technologies). CD4+T cells were incubated overnight with IMDM (Gibco) complete media (supplemented with 10% vol/vol heat-inactivated FBS, 100 U/mL penicillin-streptomycin, 2 mmol/L L-glutamine, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate). Then, 1×10^6 cells was incubated with IMDM complete media, 50 ng/mL PMA (Sigma), and 1 µg/mL ionomycin (Sigma) for 6 hours (after the first hour, Brefeldin A was added at 1:1000). After 6 hours, cells were stained with surface antibodies (PerCP-conjugated anti-CD3 [Immunotools], APC-conjugated anti-CD4 [Immunotools], PE-conjugated anti-CD45RA [Immunotools], and APC/Cyanine7-conjugated anti-CXCR5 [BioLegend]), which were fixed with Fixation/Permeabilization buffer (BD Biosciences), and incubated with Perm/Wash Buffer (BD Biosciences) overnight at 4°C. Cells were then stained with intracellular antibodies (FITC-conjugated anti-IL4 [BioLegend], Brilliant Violet 421-conjugated anti-IL13 [BioLegend], and PE/Cyanine7-conjugated anti-IFNg [BioLegend]). Cells were acquired on an Attune flow cytometer (Thermo Fisher Scientific) and analyzed using FlowJo software, version 10.8.

Western Blotting

After incubating with pooled sera from the different groups, we analyzed the intracellular activation pattern and activation with Pru p 3 in the LAD2 model. The adaptor protein LAT is critical in MC FcεRI signaling, linking the high-affinity IgE receptor to calcium influx and degranulation [40]. Western blotting was carried out as described [41,42]. LAD2 cells were sensitized with pooled sera 1:1 overnight and stimulated with 2 µg/mL of Pru p 3. The reaction was stopped at 0 seconds, 30 seconds, and 2 minutes, and cells were then lysed. Total protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's recommendations. Electrophoresis and protein blotting were performed using NuPage™ 4-12% Bis-Tris Gel, 1.5 mm*15 wells (Invitrogen), and the gel was electrotransferred to polyvinylidene difluoride membranes (Millipore). Blots were probed with anti-pTyr:HRP (BD Transduction Laboratories), rabbit anti-pLAT (Cell Signaling Technology), rabbit anti-LAT (Cell Signaling Technology), and goat anti-rabbit-HRP (Life Technologies). In all blots, proteins were visualized using enhanced chemiluminescence (WesternBright™ ECL, Advansta).

Cytokine Multiplex Assay

$CD34^+$-derived MCs from peripheral blood from patients and healthy controls and LAD2 were used to determine the mediator's release. MCs from patients (anaphylaxis and sensitized) and LAD2 were incubated overnight with pooled sera (anaphylaxis and sensitized). The next day, 1×10^5 cells was cultured in a 48-well plate and treated with 1 µg/mL Pru p 3 (Roxall) for 24 hours at 37°C. Likewise, LAD2 cells were incubated with 0.1 µg/mL biotinylated human IgE (Abbiotec) and stimulated with 0.4 µg/mL Streptavidin (Sigma). The supernatants were kept at –80°C for subsequent cytokine and chemokine measurement using the ProcartaPlex Multiplex Assay (Invitrogen), as described elsewhere [43]. In the multiplex assay, 50 µL of supernatant was combined with a panel of beads covalently bound to an antibody that recognized one of the following cytokines/chemokines: IL1-ß, IL-6, IL-8, IL-13, GM-CSF, TGF-ß, TNF-α, and CCL2.

Statistical Analysis

The statistical analysis was performed using PRISM 9 (GraphPad Software). All results are expressed as mean (SD). One-way ANOVA was used to identify significant differences (*P* value) between several experimental groups, and the *t* test was used to identify significant differences (*P* value) between the 2 groups (anaphylaxis and sensitized patients) after determining the normal distribution of the samples and variance analysis.

Results

Patient Characteristics

The characteristics of the patients recruited to obtain (*1*) serum to create pooled sera and (*2*) $CD34^+$-derived MCs and $T_{FH}13$ are listed in Tables 1 and 2, respectively.

Specific IgE (sIgE) values were higher in anaphylaxis patients than in sensitized patients, although the sIgE:tIgE was higher in sensitized patients (Table 2). The sIgG4:sIgE ratio was also higher in sensitized patients (Table 2). The tryptase level of patients was measured to rule out tryptasemia (Table 1).

Sera From Anaphylaxis Patients Induce Stronger Degranulation and PGD2 Production in CD34⁺-Derived MCs

MCs from patients and healthy volunteers were differentiated (CD117⁺/FcεRI⁺) in vitro after 7 weeks (Table S1), and their ability to degranulate to positive stimuli (PMA + ionomycin and biotinylated IgE plus streptavidin) was confirmed (Figure S1).

MCs from healthy controls, sensitized patients, and anaphylaxis patients were incubated overnight with pooled sera from all 3 groups. The serum volume was corrected by the sIgE values. Cells were then incubated with Pru p 3 for 30 minutes, and the surface expression of CD63⁺ was measured using flow cytometry.

MCs from all 3 groups showed significantly higher activation when sensitized with pooled sera from anaphylaxis patients than with pooled sera from healthy volunteers or sensitized patients (Figures 1A, B, and C, respectively). Sera from sensitized patients induced degranulation similar to that induced by sera from healthy individuals. Cells sensitized with sera that had not undergone Pru p 3 challenge were not activated (Table S2).

Next, we aimed to confirm our results by analyzing release of PGD2 under the same conditions. Again, MCs from all 3 groups had produced more PGD2 when incubated with pooled anaphylaxis sera than pooled sera from healthy volunteers or sensitized patients (Figures 2A, B, and C, respectively). However, the difference was significant only for MCs from sensitized and anaphylaxis patients, not for those from healthy individuals. Finally, a significant correlation was observed between degranulation (CD63⁺) and synthesis of PGD2 in all groups (Figure S2).

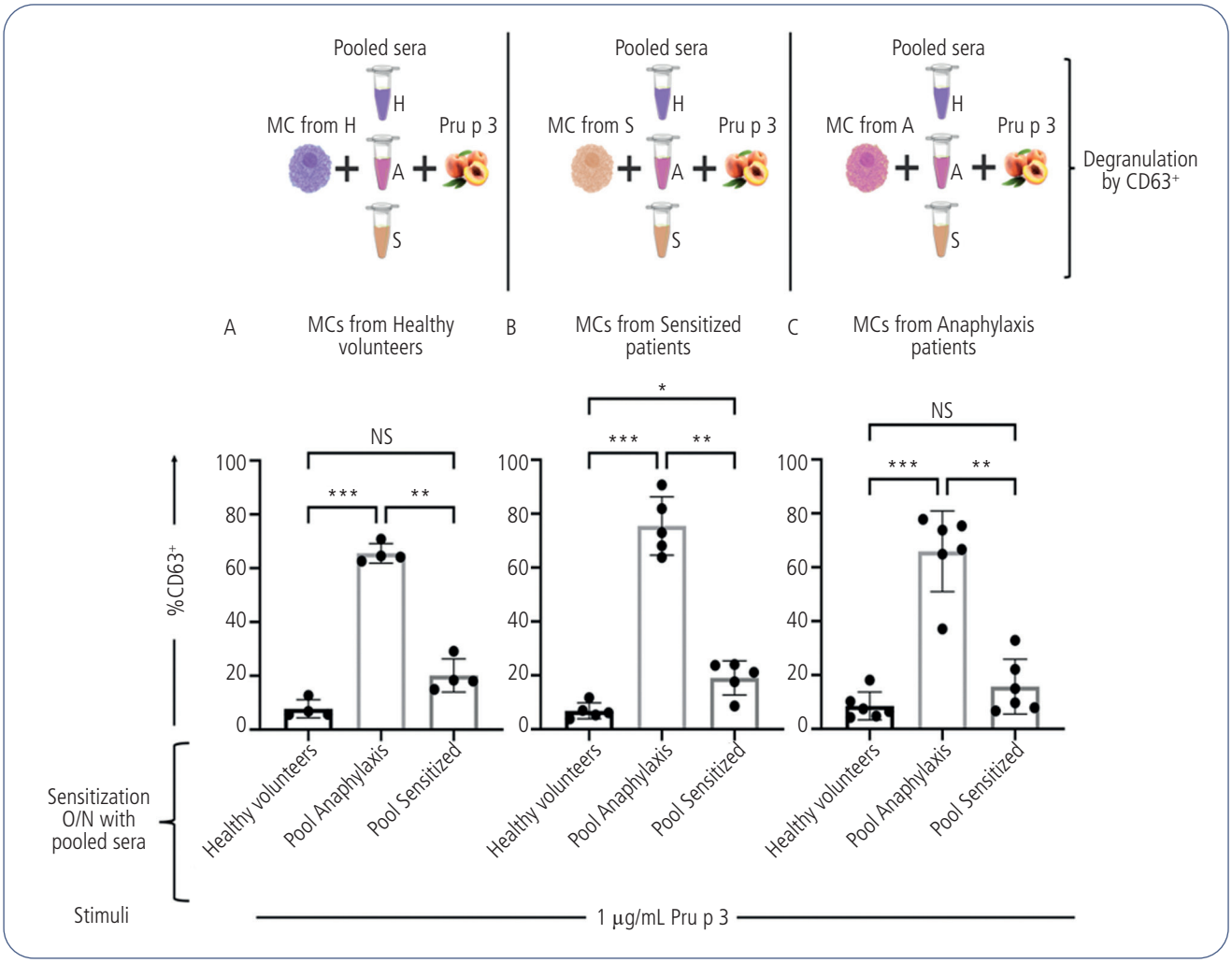

Figure 1. Sera from anaphylaxis patients induce greater MC degranulation. Degranulation measured by CD63 expression was performed with (A) MCs from healthy volunteers (n=4), (B) MCs from sensitized patients (n=5), and (C) MCs from anaphylaxis patients (n=6). MCs were sensitized overnight with pooled sera and stimulated with 1 µg/mL of Pru p 3. Results are expressed as mean (SD). *P*<.05 was considered significant. **P*<.05; ***P*<.01; ****P*<.001. MC indicates mast cell; H, healthy volunteers; A, anaphylaxis; S, sensitized; NS, nonsignificant; O/N, overnight.

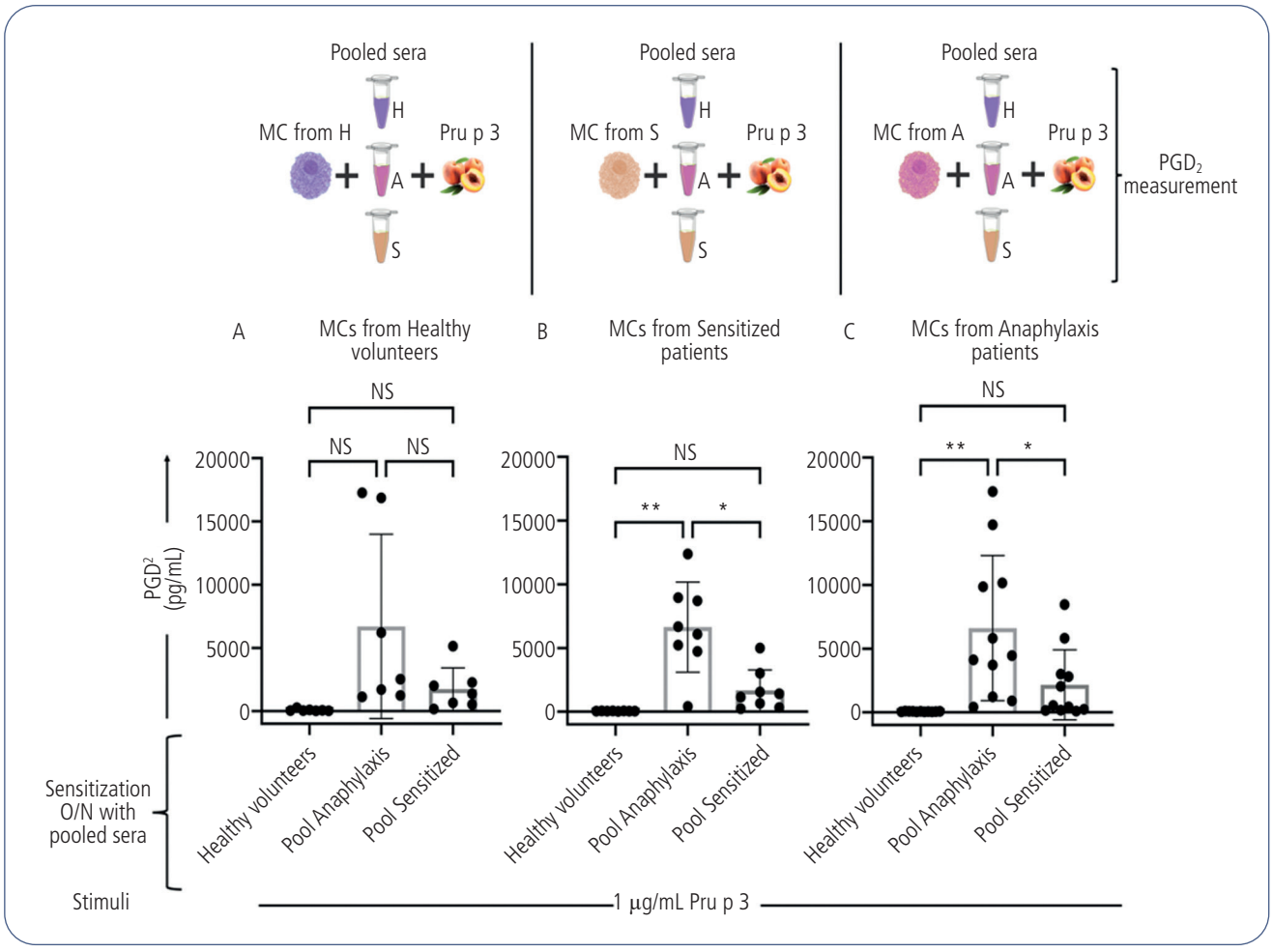

Figure 2. Sera from anaphylaxis patients induce higher PGD_2 secretion. PGD_2 secretion was studied based on (A) MCs from healthy volunteers (n=4), (B) MCs from sensitized patients (n=5), and (C) MCs from anaphylaxis patients (n=6). PGD_2 was measured in postactivation supernatant. Results are expressed as mean (SD). $P<.05$ was considered significant. *$P<.05$; **$P<.01$. MC indicates mast cells; H, healthy volunteers; A, anaphylaxis; S, sensitized; NS, nonsignificant; O/N, overnight.

Sera From Anaphylaxis Patients Induce More Robust Activation in LAD2 Cells

We performed the same experiments using LAD2 cells to correct the potential effect of the MC phenotype on activation and to reproduce our observations in a different MC model. Degranulation induced by LAD2 cells sensitized with pooled sera from the anaphylaxis patients was significantly more pronounced than that induced by cells incubated with sera from healthy and sensitized individuals (Figure 3A). As shown in Figure 3B, a phosphotyrosine protein pattern was induced, with more marked phosphorylation of LAT in anaphylaxis patients than in sensitized patients, consistent with the higher degranulation observed (Figure 3A).

$T_{FH}13$ Cells Are More Abundant in Anaphylaxis Patients

Our results show the marked ability of sIgE in the anaphylaxis pool to yield greater MC degranulation. $T_{FH}13$ cells were found to regulate the induction of high-affinity IgE [30]. Thus, we investigated the presence of the $T_{FH}13$ population in PBMCs following the gating strategy shown in Figure S3 and previously described [29].

We observed that patients from the anaphylaxis group had a significantly higher number of $T_{FH}13$ cells than the other groups. Sensitized patients presented similar results to those of healthy individuals. Indeed, we identified a significant correlation between degranulation ($CD63^+$) and presence of $T_{FH}13$ (Figure 4).

Sera From Anaphylaxis Patients Induce a More Pronounced Proinflammatory Pattern in $CD34^+$-Derived MCs and LAD2 Cells

We found that MCs from the anaphylaxis group incubated with pooled anaphylaxis sera produced more significant amounts of IL-8 and GM-CSF (Figure 5A), since they had a more pronounced proinflammatory profile than MCs from the sensitized group incubated with pooled sensitized sera. Interestingly, the MCs from the sensitized group incubated with pooled sensitized sera produced more TGF-ß and CCL2

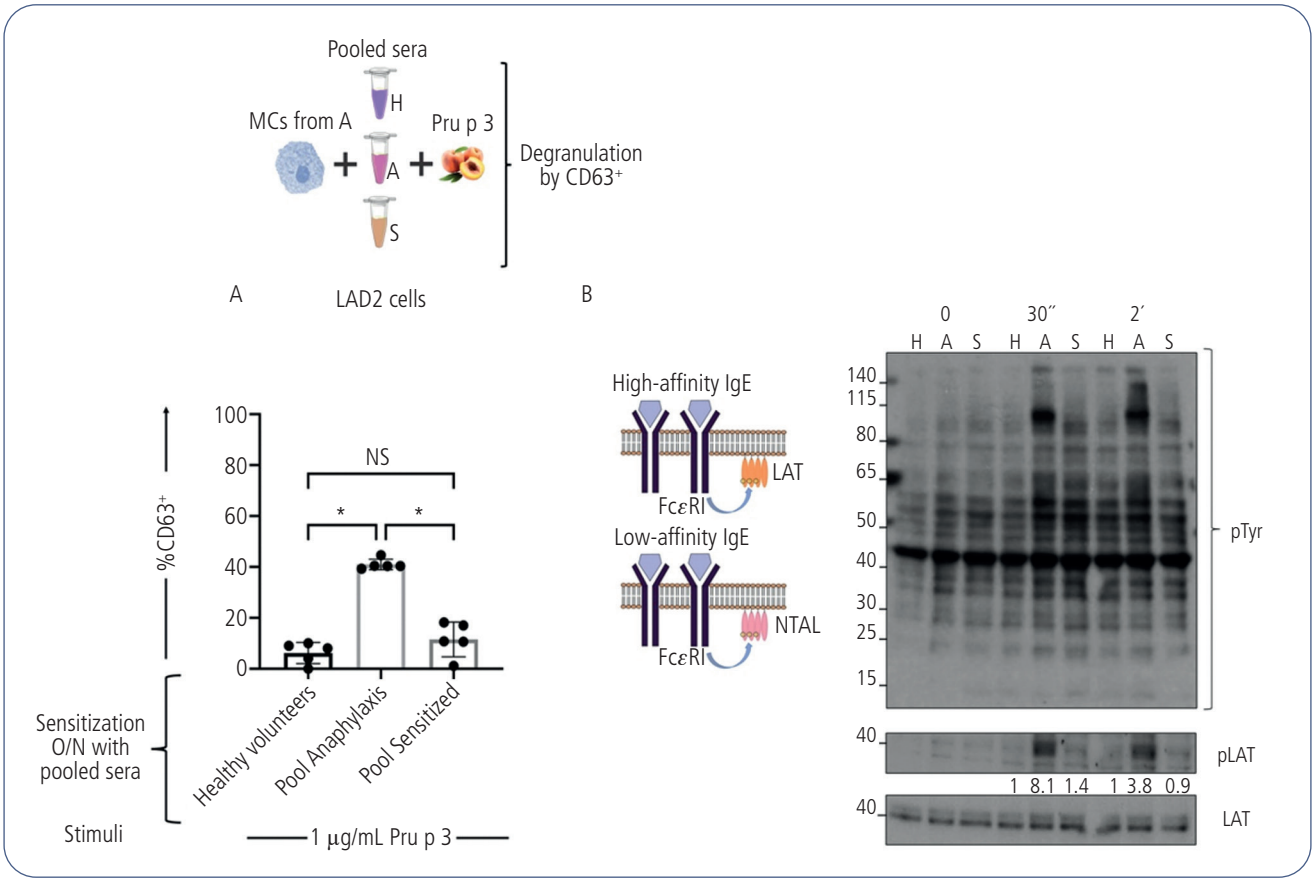

Figure 3. Anaphylaxis sera induce higher LAD2 activation. A, Degranulation measured by CD63 was performed with LAD2 cells sensitized overnight with pooled sera and stimulated with 1 µg/mL Pru p 3 for 30 seconds and 2 minutes (n=5). Results are expressed as mean (SD). *P*<.05 was considered significant. **P*<.001. B, Western blot of LAD2 cells sensitized overnight with pooled sera and stimulated with 2 µg/mL Pru p 3. pTyr indicates phosphotyrosine; pLAT, phospho-LAT; LAT, total LAT; MC, mast cell; H, healthy volunteers; A, anaphylaxis; S, sensitized; NS, nonsignificant; O/N, overnight.

(Figure 5A), inducing a more protective profile. While we did not find significant differences between groups for the other cytokines studied (IL-1β, IL-6, IL-13, and TNF-α), we did observe a trend. MCs with pooled anaphylaxis sera produce more IL-1β, IL-6, IL-13, and TNF-α than those incubated with pooled sera from sensitized patients (Figure S4).

Similarly, when LAD2 cells were incubated with pooled sera from the anaphylaxis patients or with biotinylated human IgE and activated with Pru p 3 or streptavidin, respectively, they produced more IL-8 and GM-CSF. Conversely, when LAD2 cells were incubated with pooled sera from the sensitized patients and activated with Pru p 3, they had more TGF-ß and CCL2 (Figure 5B).

Discussion

Our study showed that the MC activation profile could differentiate patients with severe reactions from patients sensitized only to LTP. Our results are consistent with those of other studies involving the MAT and peanut as the allergen [14,15,21,44]. The MAT is an in vitro diagnostic tool that combines the allergen, allergen-specific IgE, and human MCs, that is, the 3 crucial elements of the effector phase of IgE-mediated allergic responses [45]. Interestingly, in our model, we found the humoral component to be far more critical than the cellular component in inducing MC degranulation and PGD2 production, given that when LAD2 cells were used instead of $CD34^+$-derived MCs from patients, the activation/degranulation patterns were unaltered. However, LAD2 showed lower degranulation than $CD34^+$-derived MCs from patients under the same conditions, as reported elsewhere [46].

We found that sIgE levels were higher in anaphylaxis patients than in sensitized patients, possibly accounting for the more pronounced activation in the former, as reported in other studies [21]. Tam et al [45] reported that MCs from healthy donors responded to high specific IgE levels but not to low ones. However, we normalized sIgE values for each group, sensitizing all MCs with the same amount of sIgE to eliminate this potential confounding factor. Therefore, our results suggest that MC responses may depend on other humoral factors, such as the affinity of IgE for the allergen, which could vary between individuals, as previously proposed [14].

Similarly, we showed that $T_{FH}13$ cells were more abundant in patients with anaphylaxis than in those who were sensitized, indicating that they might have higher-affinity sIgE than

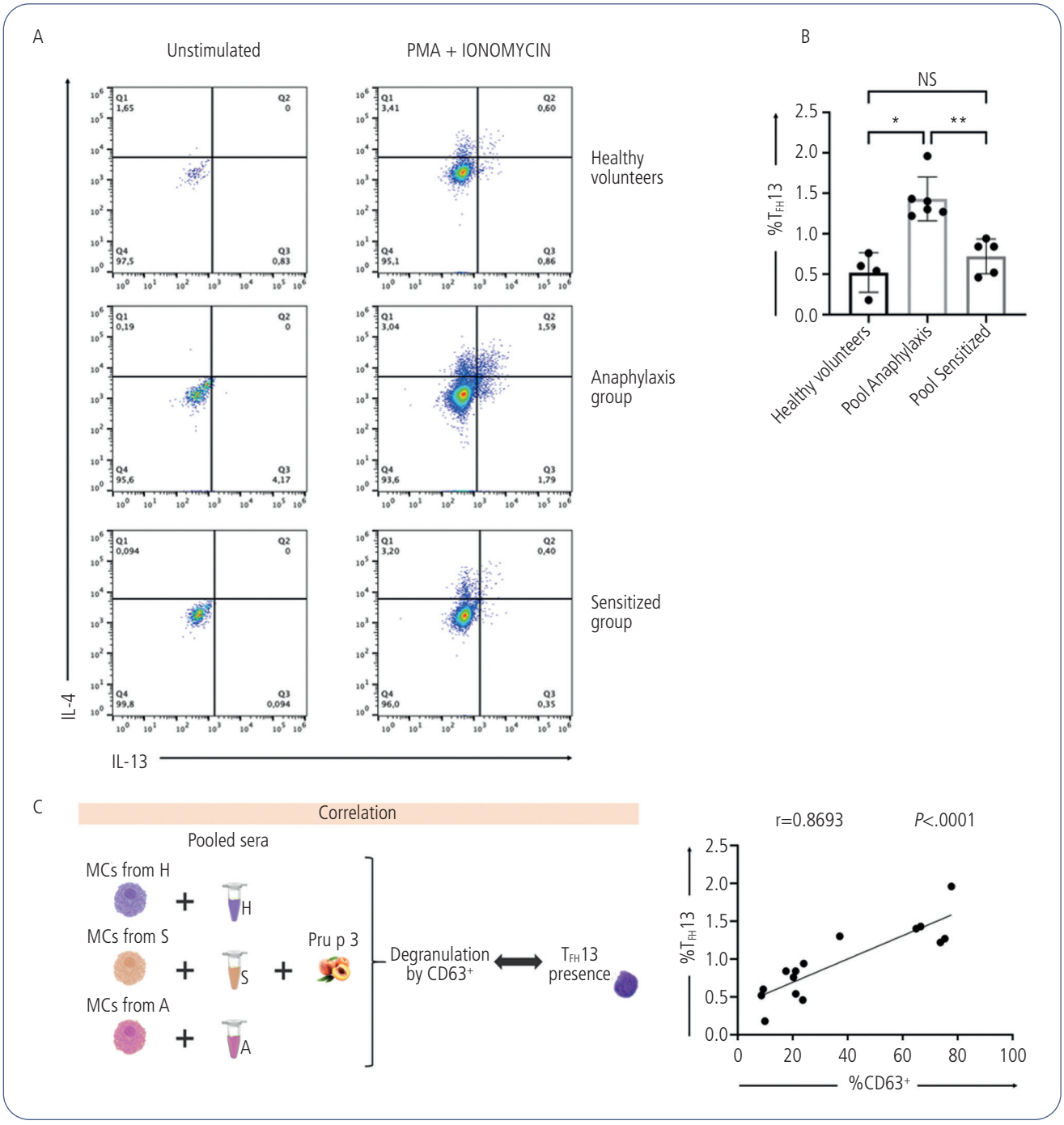

Figure 4. $T_{FH}13$ cells are more abundant in anaphylaxis patients. A, Intracellular staining of IL-4 and IL-13 in a healthy volunteer, an anaphylaxis patient, and a sensitized patient with and without PMA/ionomycin stimulation (gated as in Figure S3). B, $T_{FH}13$ cells in healthy volunteers (n=4), anaphylaxis (n=6), and sensitized patients (n=5). C, Correlation between degranulation and the percentage of $T_{FH}13$ cells. Results are expressed as mean (SD). Significance was determined using 1-way ANOVA with a Tukey multiple comparison analysis. Correlations were calculated using Pearson R values. *P*<.05 was considered significant. **P*<.001; ***P*<.01. MC, indicates mast cell; H, healthy volunteers; A, anaphylaxis; S, sensitized; NS, nonsignificant.

individuals who are merely sensitized. $T_{FH}13$ cells regulate the induction of anaphylactic IgE [30] by secreting cytokines; T_{FH} cells guide the production of specific antibody isotypes during an immune response. The high-affinity IgE prevalent in allergy cannot be induced only by T_{FH} cell–derived IL-4, although it is necessary for synthesis of IgE. Additionally, induction requires IL-13, which is produced by the $T_{FH}13$ cell population.

Furthermore, the affinity of IgE could induce different signaling patterns. As some studies have reported [23,31], high-affinity IgE can yield more robust activation of phospho-

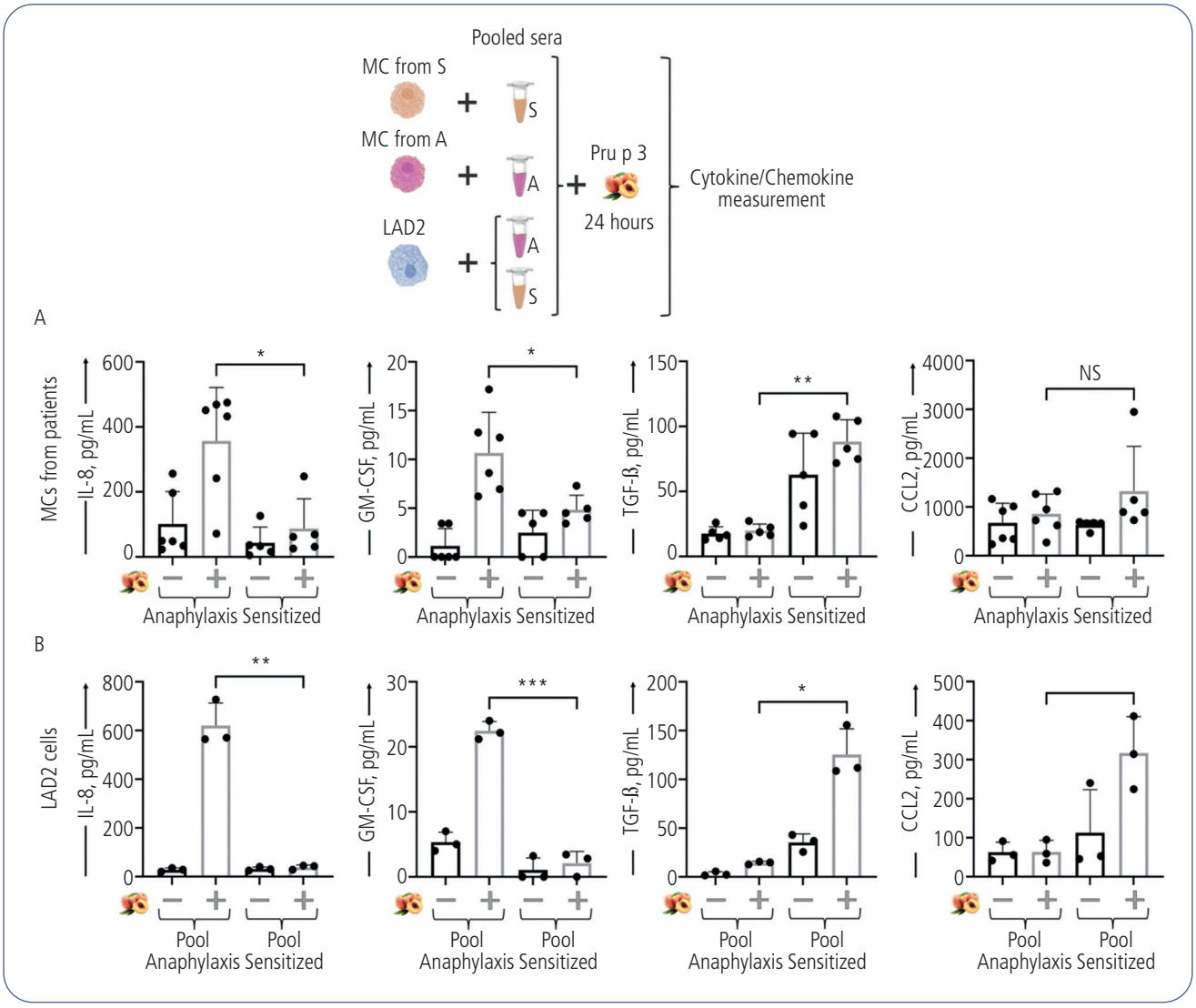

Figure 5. Sera from anaphylaxis patients induce more pronounced proinflammatory patterns. Cytokine multiplex assay was performed in (A) CD34⁺-derived MCs from anaphylaxis patients (n=6) and sensitized patients (n=5) and in (B) LAD2 cells (n=3). Results are expressed as mean (SD). Significance was determined using the t test with a Welch correction. *P*<.05 was considered significant. **P*<.05; ***P*<.01; ****P*<.0001. MC indicates mast cells; A, anaphylaxis; S, sensitized. IgEb, biotinylated human IgE; STV, streptavidin; NS, nonsignificant.

LAT1, increasing degranulation and cytokine production, with greater recruitment of neutrophils at the site of inflammation. In contrast, low-affinity IgE can induce the activation of other molecules, such as phospho-LAT2 or phospho-Fgr, thus increasing the production of chemokines such as CCL2, CCL3, and CCL4, which are monocyte- or macrophage-attracting factors. Consequently, the affinity of IgE could switch the cellular response by means of molecular signals [23]. Also interesting is epitope diversity related to the affinity of sIgE. Some studies reported a higher epitope diversity with high-affinity sIgE in allergic patients than in tolerant patients, thus correlating with the severity of allergic reactions [47-49]. In our study, we show that sera from anaphylaxis patients induce more robust activation of phospho-LAT1 in LAD2 cells and a higher amount of IL-8 and GM-CSF in CD34⁺-derived MCs and LAD2 cells, suggesting a more proinflammatory pattern. Sera from sensitized patients, on the other hand, induce a higher amount of TGF-ß and CCL2 in CD34⁺-derived MCs and LAD2 cells, indicating a more protective pattern. TGF-ß was reported to suppress MC activity and to inhibit MC FcεRI expression in mice [50,51]. These results reinforce the observation that differences in affinity of IgE and increased epitope diversity lead to different cell activation programs that may in turn induce anaphylaxis in a high-affinity context.

The cellular component of MCs may also play a role in severity [52]. A mutation in *KARS* encoding lysine tRNA synthetase, which increases microphthalmia-associated transcription factor activity, has been associated with severe anaphylaxis [39]. In contrast, STAT3 mutations have been shown to be protective in patients with hyper-IgE syndrome [53]. Moreover, an increased risk of severe anaphylaxis has been linked to hereditary variations in the copy

number of the *TPSAB1* gene, which encodes tryptase [54]. In our study, serum tryptase values below 8 µg/L in all MC donors made the presence of hereditary α-tryptasaemia unlikely.

The sIgE:tIgE ratio may be necessary for diagnostic purposes, since the clinical relevance of the sIgE level depends on its fractional relation to tIgE when determining the receptor occupancy rate of effector cells. Furthermore, the measurement of allergen-specific sIgG4 could be of additional value to indicate the development of tolerance in FA patients [55-57]. Indeed, serum IgG4 levels are higher among asymptomatic atopic patients [58]. Therefore, the IgG/IgE ratio is higher in nonatopic and asymptomatic atopic patients than in allergic patients [59,60]. We observed that anaphylaxis patients had significantly lower sIgE:tIgE and sIgG4:sIgE ratios, indicating higher relative sIgE values. However, the evidence regarding the utility of these ratios remains limited [61].

Our results are significant and consistent, considering that the number of patients is not elevated, and allow us to conclude that MC activation profile analysis may discriminate patients at risk of developing anaphylaxis from those who are merely sensitized, thus helping us to stratify risk before an oral food challenge. Indeed, identifying a larger $T_{FH}13$ population in peripheral blood may also aid this decision-making process.

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Conflicts of Interest

MPC has received honoraria for presentations from Thermo Fisher Scientific and LETI Pharma SLU. JB has received consultancy fees (advisory role) from Bial and Novartis and payment for lectures from Hal Allergy, LETI Pharma, Menarini, Novartis, and Thermo Fisher Scientifc. The remaining authors declare that they have no conflicts of interest.

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